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Correlation Between Amino Acid Exchanges in Coat Protein of TMV Mutants and the Nature of the Mutagens

B. SINGER AND H. FRAENKEL-CONRAT

Space Sciences Laboratory and Department of Molecular Biology and Virus Laboratory, University of California, Berkeley, California 94720

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This paper represents a comparative study of the action of various reagents of different mutagenicity on the messenger activity of TMV RNA, as monitored by amino acid analyses of the coat protein of 225 mutants. No such comparative data appear to be available from other laboratories.

The stable mutants with single amino acid exchanges obtained with nitrous acid, hydroxylamine, and methoxyamine showed the expected predominance of those changes which would be predicted on the basis of their reaction specificity (C → U and A → G; C → U).

The amino acid exchanges caused by bromination and alkylation indicated a greater variety of mutagenic events. Recent studies of the many reactions caused by alkylating agents, indicating that all bases may be modified at more than one site, supply an explanation for this finding.

The results of photochemical reactions which destroy bases resemble those of spontaneous mutations in giving a completely random exchange pattern.

INTRODUCTION

The studies of the chemical and the mutagenic effects of nitrous acid acting on TMV-RNA represented the first proof that point mutations are due to nucleic acid base modifications (Mundry and Gierer, 1958; Schuster and Schramm, 1958; Schuster and Vielmetter, 1961). The specific effects of many different chemicals on polynucleotides have since been intensely studied. It has become evident that the predominant reaction need not represent the biologically effective one. Particularly with alkylating reagents minor reactions have recently been discovered that may actually be responsible for the mutagenic and/or carcinogenic efficiency of the respective reagent.

In our laboratory we have been conducting parallel studies on the chemistry of many mutagens with particular reference to the formation of minor derivatives, and also mutating TMV or TMV-RNA with these same reagents and measuring the mutation frequency, and analyzing the mutant coat proteins. We now present the results of amino acid analysis of 225 mutants of TMV obtained with a variety of modifying reactions, and the attempts to correlate the observed amino acid exchanges with what is presently known of the chemistry of these reactions.

MATERIALS AND METHODS

TMV or its RNA, depending on the reagent, was reacted with HNO_2 according to Mundry and Gierer (1958); hydroxylamine (HA) and methoxyamine (MA) according to Fraenkel-Conrat and Singer (1972); nitrosoguanidine, dimethyl sulfate, and methyl methanesulfonate according to Singer and Fraenkel-Conrat (1969a,b); and aqueous bromine or *N*-bromosuccinimide according to Brammer (1963) and Means and Fraenkel-Conrat (1971).

The photocatalyzed reactions of TMV-RNA with iron salts or dyes were according to Singer and Fraenkel-Conrat (1965,

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1966). Very brief treatment of TMV-RNA with snake venom phosphodiesterase was according to Singer and Fraenkel-Conrat (1963). The chemical elimination of the terminal nucleoside of TMV-RNA was according to Steinschneider and Fraenkel-Conrat (1966).

Mutational reactions were generally performed on TMV-RNA which was then reconstituted with TMV protein (Fraenkel-Conrat and Singer, 1959) before inoculation to plants. Nitrosoguanidine is highly mutagenic only when TMV-RNA is in the virus, and for this reason only intact TMV was used.

The method for the determination of infectivity and mutation frequency are given by Singer and Fraenkel-Conrat (1969c). Tables of relative mutagenicity have been published by Singer and Fraenkel-Conrat (1969c,d).

The usual methods were used for obtaining purified preparation of TMV mutants by differential centrifugation and of the protein by the acetic acid method (Tsugita and Fraenkel-Conrat, 1962). The techniques of hydrolysis and amino acid analysis were also the same as in earlier studies. Analytical data on isoleucine, leucine, and valine were based on 72-hr hydrolysis, and those for serine and threonine on extrapolation to 0 time from the 24 and 72 hr analytical data.

RESULTS

The method of selection of mutants for study was, for practical reasons, the isolation of those giving local lesions on *N. sylvestris*.[1] The process of freeing such mutants from wild-type virions was by repeated transfer to the same host and/or to *Nicotiana tabacum* var. Xanthi nc. Only a small percentage of the mutants obtained by chemical treatment of TMV or its RNA survived such repeated transfers, which is in distinct contrast to the spontaneous mutants or contaminants present in untreated TMV, which were completely stable during such repeated transfers. About 10% of the viable mutants derived from chemical modification were unstable in the sense that they changed in symptomology and/or amino acid composition upon repeated transfers. These are believed to represent products of secondary spontaneous mutations of low viability mutants, and they are grouped together with previously discussed mutants differing grossly from wild-type in coat protein structure and resembling other TMV-groups (e.g., group B = Dahlemense, etc.) (Tsugita and Fraenkel-Conrat, 1962; Tsugita, 1962).

Of the 197 stable mutants here reported on 12 showed exchanges of 2 or 3 different amino acids and these are not included in the analysis of the data, because their codon assignment is not generally unambiguous. One third of the remaining mutants showed no amino acid exchanges in the coat protein; these presumably carry exchanges in other, yet undetermined, virus-coded proteins.

The observed amino acid exchanges in the remaining 117 mutants, and the frequencies of these exchanges as obtained after various mutagen treatments, are listed on Table 1. Most of these, but particularly the unexpected ones (other than C → U or A → G) and those giving ambiguous or unstoichiometric analyses (reproducibly $\gtrsim 0.25$ above or below an integer number of amino acid residues per mole) were regrown and the protein of the progeny virus was reanalyzed 2–4 times, a procedure which led occasionally to the detection of unstable mutants, as discussed above and not included in the table. Table 2 summarizes all the definitive data. The frequencies of obtaining exchanges other than C → U or A → G are listed on Table 3.

Amino acid exchanges resulting from nitrous acid mutagenesis can be accounted for to 89% by C → U and A → G modifications, in good agreement with earlier data from the laboratories of Fraenkel-Conrat and Wittman (summarized by Funatsu and Fraenkel-Conrat, 1964), and in support of previous conclusions that the deamination of cytosine and adenine leads to miscoding, but not that of guanine (Schuster and Vielmetter, 1961). Hydrox-

[1] Earlier studies from this laboratory included mutants which differed only in systemic symptomology from wild type TMV. These mutants are not included in the present summary.

TABLE 1

FREQUENCY OF AMINO ACID EXCHANGES IN COAT PROTEIN OF PROGENY VIRUS OF TMV TREATED WITH VARIOUS MUTAGENS

Amino acid exchange[b]	Possible codon exchange	Modification reaction[a]					
		HNO_2	HA, MA	Alkyl	Br_2	Photo chem.	Term. elim.
Ala → Ser	G → U			1			
Ala → Val	C → U			2			
Arg → Gly	A → G	5		1	1		
Arg → Leu	G → U				2		
Arg → Lys	G → A	1					
Asn → Ser	A → G	5			1		
Asp → Gly	A → G			1			
Gln → Arg	A → G	1					
Gln → His	Pu → Py					4	3
Glu → Gly	A → G	1					
Ile → Asn	U → A					1	1
Ile → Thr	U → C		1	4	2	1	2
Ile → Val	A → G	4	1	4	2	1	2
Leu → Thr	2 exch.			1			
Pro → Leu	C → U	4	1	3	5		
Pro → Ser	C → U			1		2	
Ser → Gly	A → G				1		
Ser → Leu	C → U			1		1	
Ser → Phe	C → U	9	7	7	3		1
Ser → Pro	U → C						1
Thr → Ala	A → G	1					
Thr → Ile	C → U	2					
Tyr → Phe	A → U			1			
Val → Ala	U → C			1			
Val → Ile	G → A	2					
Val → Met	G → A	1		1		1	2
Val → Phe	G → U			1			
Val → Pro	2 exch.					1	

[a] The modification reactions are abbreviated as follows: nitrous acid, HNO_2; hydroxylamine and methoxyamine, HA and MA; alkylation including nitrosoguanidine, dimethyl sulfate, and methyl methane sulfonate, Alkyl; *N*-bromosuccinimide and bromine water, Br_2; dye and metal catalyzed photochemical reactions, Photo-chem; brief snake venom phosphodiesterase treatment or stepwise chemical elimination, Term-elim.

[b] In the cases of exchanges involving aspartic or glutamic acids or their amides, the one accounting for the observed exchange on the basis of a single nucleotide replacement is arbitrarily listed.

ylamine and methoxyamine primarily substitute the exocyclic amino group of cytosine in ribopolynucleotides (Fraenkel-Conrat and Singer, 1972), enabling it to pair like uracil (Brown *et al.*, 1968; Singer and Fraenkel-Conrat, 1970), and the observed amino acid exchanges are in good accord with this mechanism (Table 1).

The effects of alkylating agents on RNA and DNA is actively being studied in several laboratories, and is reviewed by Singer (1974). There is substantial agreement that the predominant reaction, the 7-alkylation of guanine is not responsible for the carcinogenic effects of many alkylating agents (Swann and Magee, 1968, 1971; Schoental, 1969; Ross *et al.*, 1971; Lijinsky *et al.*, 1972; O'Connor *et al.*, 1972; Kleihues and Magee, 1973; Craddock, 1973) and is not per se mutagenic (Ludlum, 1970). In the specific case of nitrosoguanidine acting on intact tobacco mosaic virions, methylation of cytosine could be correlated with mutagenicity (Singer and Fraenkel-Conrat, 1969b,c, 1970). On the other hand, it is suggested by recent data

TABLE 2

SUMMARY OF MUTANTS OF TMV PRODUCED WITH VARIOUS MUTAGENS AND CODON EXCHANGES DERIVED FROM AMINO ACID ANALYSIS OF THEIR COAT PROTEIN

Modification reaction[a]	Number of mutants[b]					Codon exchanges[c]					
	Total	Unstable	Multiple exchanges	No exchange	Single coat protein exchange	Number			%		
						C → U	A → G	Other	C → U	A → G	Other
HNO_2	65	11	7	11	36	15	17	4	42	47	11
HA, MA	23	2	1	10	10	8	1	1	80	10	10
Alkylating agents	73	9	2	25	30	14	6	10	46	20	33
Br_2	28	4	1	6	17	8	5	4	47	29	24
Photochemical reactions	20	—	—	8	12	3	1	8	25	8	67
SV-DE or term. chem./elim.	16	2	1	1	12	1	2	9	8	17	75
Total	225	28	12	61	117	49	32	36			

[a] References for the reaction conditions are given in Methods and Materials section. Fifty-eight of the alkylated mutants were obtained with nitrosoguanidine treatment of TMV. The rest were obtained with dimethyl sulfate or methyl methane sulfonate treatment of TMV-RNA.

[b] The total figure refers to the number of separate mutants and does not include reisolated mutants. Unstable mutants are those which upon reisolation exhibit changed symptomology and/or changed amino acid composition. Multiple exchanges refers to more than 1 exchange, except when 2 identical amino acids are exchanged for two other identical amino acids (e.g., 2 Ser → 2 Phe).

[c] Codon exchanges are given only for those mutants listed in the category of "coat protein exchange," and do not include those with multiple exchanges (except as noted in footnote[b]) or unstable mutants. Codon exchanges listed as "Other" are given in detail in Table 3. All amino acid exchanges are given in detail in Table 1.

TABLE 3

FREQUENCY OF CODON EXCHANGES OTHER THAN C → U AND A → G[a]

Codon exchange	Modification reaction[b]						Total exchanges
	HNO_2	HA, MA	Alkyl	Br_2	Photochem.	Term. elim.	
Pu → Pu							8
G → A	4		1		1	2	
Pyr → Pyr							12
U → C		1	5	2	1	3	
Pu → Pyr					4	3	12
G → U			2	2			
A → U			1				
Pyr → Pu							2
U → A					1	1	
double exchange			1		1		2

[a] Data from Table 1.

[b] Abbreviations are the same as in Table 1.

on alkylation of DNA and RNA with certain nitroso compounds that the substitution of the O^6 (O'Connor *et al.*, 1972; Lawley and Shah, 1972; Craddock, 1973) or possibly the 3 position of guanine (Lawley *et al.*, 1971) may be correlated with the high carcinogenicity of these compounds. The amino acid data obtained with alkylation mutants of TMV (Table 1), largely due to nitrosoguanidine treatment of the virus, since this is the only effective alkyl-mutagenizing method, support the predominant modification of cytosine. The relatively large number of C → U exchanges (Table 2), however, would not have been predicted, since none of the chemical effects of alkylating agents would be expected to reverse the pairing potential of the nucleotides, but rather render the base nonspecific, as demonstrated for 3 alkylcytosine (Singer and Fraenkel-Conrat, 1970; Ludlum, 1970, 1971; Ludlum and Magee, 1972), and O^6-alkylguanine (Gerchman and Ludlum, 1973).

Bromination of RNA causes primarily guanine and cytosine modification (Bram-

mer, 1963). However, only the reaction with cytosine has been studied in detail, and, when brominated poly(C) was used as template for RNA polymerase, it was found to pair with A as well as G, indicating that this reaction is mutagenic and that modified cytosine acts like uracil (Means and Fraenkel-Conrat, 1971). (Surprisingly, however, neither poly-5-bromocytidylic acid nor poly-5-hydroxycytidylic acid mispair in this system.) The data in Table 2 do show a preponderance of C → U exchanges in the coat protein of bromine-induced mutants. However, there are a number of other exchanges (G → U, U → C) (Table 3).

When RNA is illuminated with visible light in the presence of proflavin or thiopyronin, guanine is preferentially photodecomposed (Singer and Fraenkel-Conrat, 1966). Illumination of RNA in the presence of Fe^{2+} or Fe^{3+} leads to general destruction of bases (Singer and Fraenkel-Conrat, 1965). Neither the metal- nor dye-catalyzed reaction causes a high mutation frequency, but those mutants found would be expected to show a random pattern of amino acid exchanges and the data indicate that 6 of the 12 possible codon exchanges are found in the 12 mutants analyzed (Tables 2 and 3).

Occasional mutants with amino acid exchanges observed after treating RNA with the methods of stepwise degradation or by minimal snake venom diesterase treatment, both not expected to be mutagenic, showed a similarly random pattern, in support of the belief that these are spontaneous mutants.

DISCUSSION

Nitrous acid is the only reagent that is able to effect a direct base change, C → U. The action of nitrous acid on adenine, and that of hydroxylamine or methoxyamine on cytosine lead to products that favor the alternate tautomeric state (hypoxanthine and N^4-hydroxy- or N^4-methoxycytosine) and thus are effective mutagens.

Alkylating agents are capable of a wide variety of base modifications besides the predominant 7-alkylation of guanine. None of these products of alkylation would be able preferentially to pair in opposite manner. The alkylation of the 7 position of guanine appears to be without biological consequence, except due to its tendency to become depurinated in DNA. The alkylation of the 3 position of cytosine and of the O^6 of guanine make these unable to form typical pairs of H-bonds. These two modifications have been shown to mispair in random fashion. The methylation of cytosine was shown to be mutagenic in TMV, and the methylation of the O^6 of guanine was suggested to account for the carcinogenic effects of certain nitrosoalkyl compounds. It has further been suggested that mispairing may be due to 3-methylguanine, 3-methylthymine, and O^4-methylthymine, all recently found to be minor products in nucleic acids treated with the carcinogen, nitrosomethylurea (Lawley *et al.*, 1971; Lawley and Shah, 1972; Lawley *et al.*, 1973). Certain ethylating agents, also found to be carcinogenic or mutagenic, have additional specificities, and substitute guanosine on the 1 position (Singer, 1972), and cytidine and adenosine on the exocyclic amino group (Sun and Singer, 1974; Singer *et al.*, 1974).

Alkylating agents, with their potential for modifying bases on many sites, none of which appear to be specifically mutagenic, might exert their powerful biological effects through multiple synergistic action.

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